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14. ABSTRACT
The objective of this investigation is to examine the protective effects of prolactin (PRL) against anti-cancer drugs. Three specific aims were formulated: 1) To characterize the protective effects of PRL against several anticancer drugs, 2) To determine anti-apoptotic mechanism(s), and 3) To examine the protective effects of PRL in vivo. Good progress has been made in all objectives as follows. First, we have shown the protective effects of PRL against taxol, cisplatin and vinblastine in more than one breast cancer cell line. Two, we defined the window of time for the action of PRL. Three, we have established qualitative and quantitative assays for apoptosis. Due to taxol cytotoxicity, initial in vivo studies were unsuccessful. Future research will be redirected to an emphasis on anti-cancer drugs other than taxol and the use of SCID rather than PRL-/- mice.

15. SUBJECT TERMS
Prolactin, chemotherapy, breast cancer

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Introduction

Taxol, cisplatin and doxorubicin are common chemotherapeutic agents used in the treatment of breast cancer. Unfortunately, partial or complete resistance to these drugs is a major problem facing patients. Prolactin (PRL) is a hormone produced by the pituitary gland whose main target it the breast where it acts as a mitogen/survival factor. Additionally, PRL is produced by breast cancer cells, and expression of the PRL receptor (PRLR) is higher in malignant breast tissue than adjacent normal tissue. Our laboratory generated PRL over-expressing breast cancer cells that show increased tumor growth in nude mice and had higher expression of the anti-apoptotic protein Bcl-2 than controls (1). These data suggest that PRL plays a role in both mitogenesis and survival of breast cancer cells. PRL has previously been shown to be a survival factor in both prostate and breast cancer cells (2,3). Based on these data, we hypothesized that PRL antagonizes chemotherapeutic agents by altering the expression of endogenous survival proteins. This hypothesis, when proven, should provide an explanation for the ineffectiveness of some anti-cancer drugs in patients with elevated PRL serum levels, high PRLR expression or increased breast PRL synthesis.

Body

Hypothesis: PRL confers chemoresistance in breast cancer by affecting anti-apoptotic proteins. The following questions will be addressed: a) do the protective effects of PRL cover a broad range of anticancer drugs and cell types? b) what are the mechanisms by which PRL exerts its anti-apoptotic activity?, and c) can protection by PRL against taxol-induced tumor suppression be validated in an animal model of breast cancer?

The basis for this hypothesis was our preliminary data which utilized several breast cancer cell lines and chemotherapeutic agents. Low doses of PRL (1-25 ng/ml) completely antagonized the cytotoxic effects of both taxol and cisplatin in MDA-MB-468 cells, although the two drugs induce cell death by different mechanisms. For *in vivo* studies, we generated a tetracycline-inducible 468 cell line with secretable PRL. Exposure of these cells to PRL, via incubation with doxycycline (Dox), protected them from taxol cytotoxicity. These data formed the basis for further studies investigating the role of PRL in chemoresistance in breast cancer.

The specific aims were as follows:

Specific aim 1: To characterize the protective effects of PRL from apoptosis induced by several anticancer drugs.

Specific aim 2: To determine anti-apoptotic mechanism(s) by which PRL antagonizes taxol cytotoxicity in 468 cells.

Specific aim 3: To examine the protective effects of PRL against taxol cytotoxicity *in vivo*.

Rationale: Taxol is a common chemotherapeutic agent used for treating breast cancer patients. It acts by binding to microtubules, resulting in cell cycle arrest and eventual apoptosis. Additional anti-cancer drugs utilized in breast cancer treatment include another microtubule altering drug, vinblastine, and DNA-damaging agents, such as cisplatin and doxorubicin. Yet, regardless of the drug utilized, many patients are either partially or completely resistant to

treatment. Proposed mechanisms of resistance include increased expression of pro-survival proteins within the cells, extrusion of drugs from the cells by the p-glycoprotein transporter as well as defective DNA repair mechanisms. It is, therefore, important to establish that protection by PRL is not just against one type of anti-cancer drug.

Key Research Accomplishments

1. Responsiveness of MDA-MB-231 cells to taxol and partial protective effects of PRL

First, we wished to demonstrate that the protective effects of PRL are not unique to 468 cells. As shown in **Fig 1**, taxol at concentrations ranging from 0.2 to 625 ng/ml decreased MDA-MB-231 cell number in a dose-dependent manner. However, the efficacy of inhibition by taxol was not as strong as that observed in 468 cells, indicating a higher resistance to taxol treatment in 231 cells. Indeed, treatment of 231 cells with 2, 10 or 50 ng/ml PRL provided only partial protection against the lower dose of taxol, and were less effective, albeit still significant, against the higher doses of taxol (**Fig 2**). We concluded that MDA-MB-231 cells are not the optimal experimental cell model with which to examine the anti-cytotoxic effects of PRL.

2. Time-dependent studies on the protective effects of PRL in 468 cells

Our next objective was to determine the length of time which is required for PRL to exert its effects, since all our studies thus far involved a 24 hrs pre-incubation with PRL. In this case, we used vinblastine and 468 cells. **Fig 3** clearly shows that 25 ng/ml of vinblastine reduced 468 cell viability by approximately 50%. Pre-treatment of the cells with a low dose (25 ng/ml) of PRL 1-24 hrs prior to vinblastine exposure completely antagonized its cytotoxic effects. Pre-treatment with PRL either 8 or 24 hrs prior to vinblastine increased cell number above control levels. Similar data were obtained with taxol (data not shown). These results suggest a rapid process by which PRL exerts its protective effects.

3. Protection against cisplatin cytotoxicity in 468 cells with inducible PRL

We further characterized our tetracycline-inducible PRL 468 cells (tet-on 468), previously shown to partially antagonize taxol cytotoxicity. In this case, we used cisplatin. As shown in **Fig 4**, cisplatin at the range of 100 to 400 ng/ml caused a marked dose-dependent suppression of tet-on 468 cell viability down to 25% of control. The cells were then pre-incubated for 24 hrs with 0.25 μg Dox, a dose that was previously shown to release PRL. As evident in **Fig 4**, PRL released by Dox treatment caused an almost complete reversal of the cytotoxic effect of the low dose of cisplatin, but was less effective against the higher doses. These results confirmed that inducible PRL provides protection against more than one cytotoxic agent.

4. Effects of extracellular matrix on cell responsiveness to PRL

There is an increased recognition that different extracellular matrices (ECM) alter the effects of PRL on breast cancer cells. Indeed, PRL has been reported to induce focal adhesion kinase (FAK) signaling upon interactions with ECM proteins (4). To this end, we coated the plates either with collagen IV, fibronectin or a combination of the two. Wild type 468 cells were treated

with PRL 24 hrs before addition of 100 ng/ml cisplatin and cell viability was determined after 5 days. In wells containing no matrix, PRL increased cell viability by 40% compared to cisplatin alone (**Fig 5**). Cell viability was increased by 45% in cells plated on either fibronectin or collagen. Although the relative differences appear to be small, the overall increase in cell number (as judged by optical density) suggests that cells prefer the presence of a matrix. Therefore, we plan on examining other matrices such as laminin and Matrigel. The importance of the ECM should also be taken into consideration for mechanistic studies.

5. Temporary loss of PRL responsiveness

Upon completion of the time-dependent studies and the characterization of PRL protection against anti-cancer drugs other than taxol, we were ready to begin our apoptosis studies. At this point in time, however, the protection rendered by PRL was lost. Regardless of the cell line or chemotherapeutic agent used, PRL no longer antagonized anti-cancer cytotoxicity. We spent about 6 months trying to resolve this issue. Our attempts to revive the PRL protection included: a) testing new batches of PRL, both pituitary-derived and recombinant, b) purchasing new cells from ATCC, c) varying the amounts of FBS and/or charcoal stripped serum (CSS) in the media during normal cell growth and anti-cancer drug treatment, and d) testing various CSS starvation times. When all of the above failed, we began testing new batches of fetal bovine serum (FBS). Finally, we were able to identify batches of FBS from 2-3 companies that were compatible with cell responsiveness to PRL with either taxol, cisplatin and vinblastine. We believe that the presence of various levels of lactogenic hormones in the different FBS batches are responsible for the altered PRL responsiveness.

6. Studies with immunodeficient mice

In parallel with the *in vitro* studies we begun initial *in vivo* studies. Since the Rag2-KO ($PRL^{-/-}$ or $PRL^{+/-}$) mice were still being characterized, we used SCID mice. Thirty mice were randomly divided into the following groups: a) control, b) taxol treatment, c) dox, and d) dox + taxol. The tet-on 268 cells (1×10^7) were inoculated into the mammary fat pads of all mice. Designated mice were given food containing dox immediately following cell injection and for the duration of the studies. Tumors were measured once weekly and when tumor volume reached 100 mm^3, mice were given weekly injections of 12 mg/kg taxol. We observed no decrease in tumor volume with this dose of taxol and subsequently increased the dose to 15 mg/kg taxol. Unfortunately, many of the animals experienced extreme drug toxicity, causing their eventual death. Additionally, while there was a trend towards increased tumor volume in the dox treated animals, the large variability negated statistical significance. Several key changes need to be made prior to the next *in vivo* study. First, due to the extreme cytotoxic effects of taxol, another chemotherapeutic agent, such as cisplatin, will be utilized. Ondeed, we have already confirmed *in vitro* that dox treatment protects tet-on cells from a low dose of cisplatin treatment (**Fig 3**). Second, preliminary experiments will determine drug dosages that decrease tumor growth without compromising animal health. Third, we will ensure that dox concentration in the food is high enough to induce PRL secretion. Alternatively, it can be added to the drinking water.

7. Establishment of a quantitative caspase 3 assay

Focus has now shifted towards elucidating the mechanism by which the PRL protection occurs. A reduction in cell viability/number could be due to a number of processes, including apoptosis,

cell cycle arrest, necrosis, autophagy or mitotic catastrophe. However, it is generally accepted that chemotherapeutic agents reduce cell number by inducing apoptosis. Caspase 3 cleavage is considered a common, though not an exclusive, mechanism that results in apoptosis. Our first goal was to determine whether cisplatin induced caspase 3 cleavage in 468 cells, using Western blot analysis. As shown in **Fig 6**, cleavage is observed after 24 or 48 hrs following treatment with 100 ng/ml or 400 ng/ml cisplatin. Ongoing studies are now examining caspase 3 activity in response to vinblastine and doxorubicin. Currently, we are optimizing a fluorimetric caspase 3 assay (**Fig 7**), which requires a lower number of cells than western blotting and hence enables a larger number of treatments. In addition, it provides a much better quantitative evaluation than Western blotting.

Reportable Outcomes

1. The most important changes to our next *in vivo* studies will be the use of SCID mice instead of the Rag2-KO ($PRL^{+/-}$ or $PRL^{-/-}$) mice. Initially, the Rag2-KO mice were going to allow us to determine the contributions of circulating vs. breast-produced PRL on tumor growth in the presence and absence of chemotherapeutic agents. However, a recent report by Utama *et al* (5), published after we submitted our proposal, has convincingly shown that human PRL receptors are insensitive to mouse PRL. Their data were obtained with breast cancer cells and xenografts. Based on this discovery, the Rag2-KO mice no longer offer an advantage over SCID mice.

2. While there was a delay in the mechanistic portion of the studies due to the loss of PRL protection, this problem is now resolved. We have learned there is a crucial balance between lactogenic hormones in the FBS and proper cell starvation times in order to observe the effects of PRL. This should also hold true for others investigating the role of PRL in cancer. Special care is now being taken to properly tests new lots of FBS when the need arises. Our studies also suggest the ECM may play an important role in cell response and is being further investigated.

3. All future experiments will examine PRL antagonism of cisplatin, vinblastine or doxorubicin rather than taxol. We found taxol to be very difficult to work with, not only because of its high toxicity in our mice, but also in cell models because of its water insolubility. All of our breast cancer cells are strongly inhibited by the taxol vehicle (DMSO) even at very low concentrations, making it difficult to have appropriate controls. The three water soluble drugs mentioned above present much better options.

4. The rapid cleavage of caspase 3 cleavage should be ideal for inhibitor studies, including the determination of PRL effects in combination with the anti-cancer drugs in the presence/absence of JAK, MAPK and PI3K inhibitors as specified in the original proposal. Additional apoptosis assays may include DNA fragmentation, cytochrome c release and/or TUNEL staining. Ultimately, the expression/phosphorylation of various pro and anti-apoptotic Bcl-2 family members will be analyzed in the 468 cells in response to anti-cancer drugs and/or PRL.

Conclusions

Although there were few set backs, we are progressing well in establishing the role of PRL in imparting chemoresistance in breast cancer. Future studies should examine whether certain drug

combinations or increased dosages can overcome the tumor promoting effects of PRL. The results of this research should inspire innovative clinical interventions since protection from drug-induced tumor shrinkage by PRL can explain chemoresistance in certain patients. Such patients may have slightly elevated serum PRL levels, increased breast PRL production or increased PRLR expression. Testing PRL serum levels or breast PRL receptor expression in biopsy specimens may predict chemosensitivity. Moreover, agents that block PRL production or action should improve the efficacy of chemotherapy. Importantly, the involvement of PRL in chemoresistance may extend to other types of cancers.

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Figure Legends

Fig 1: Taxol induces dose-dependent decrease in cell viability in MDA-MB-231 cells. Cells were plated in DMEM + 1% CSS and treated with increasing concentrations of taxol (0-625 ng/ml) for 4 days. Cell viability was determined by MTT.

Fig 2: PRL partially protects MDA-MB-231 cells from taxol cytotoxicity. Cells were treated with increasing doses of PRL (2-50 ng/ml) 24 hrs prior to taxol (5 or 125 ng/ml) exposure. See Fig 1 for other details.

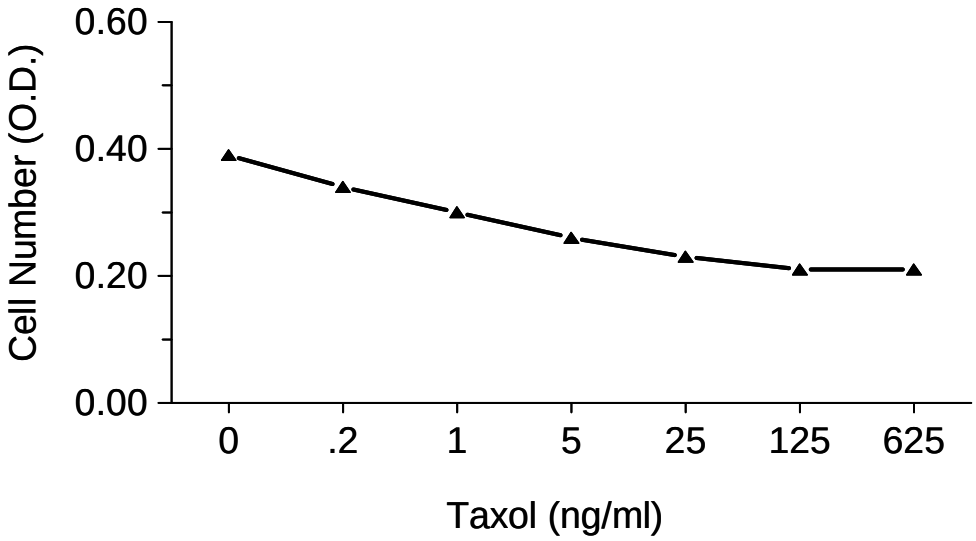
Fig 3: Time-dependent protective effect of PRL against vinblastine cytotoxicity in 468 cells. Cells were treated with PRL (25 ng/ml) 1-24 hrs prior to vinblastine (25 ng/ml) exposure. See Fig 1 for other details.

Fig 4: PRL, release by doxycycline (Dox), protects 468 tet-on cells from a low dose of cisplatin. Cells were exposed to Dox (.25 µg/ml) 24 hrs prior to cisplatin (100-400 ng/ml) treatment. See Fig 1 for other details.

Fig 5: Extracellular matrix has only small influence on PRL protection. Wells were coated with collagen, fibronectin or a combination of the two prior to plating 468 cells in DMEM + 1% CSS. Cells were treated with PRL (25 ng/ml) for 24 hrs prior to cisplatin (100 ng/ml) exposure. Viability was assessed by the MTT assay after 5 days.

Fig 6: Cisplatin induces caspase 3 cleavage in 468 cells. Cells were treated with cisplatin (Cis; 100 or 400 ng/ml) for 24 or 48 hrs. Western blotting shows both full length caspase 3 (C3 at 35 kDa and cleaved caspase 3 (CC3) at both 17 and 19 kDa.

Fig 7: Cleavage of caspase 3 in 468 cells by cisplatin, as determined by a fluorimetric assay. Cells were incubated with increasing doses of cisplatin (0-800 ng/ml) for 24 hrs. Cell lysates were incubated with a synthetic peptide substrate, DEVD-AMC for 45 min. Free AMC was quantified using a fluorescent plate reader.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0.00
Cell Number (O.D.)
0
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1
5
25
125
625
Taxol (ng/ml)

Fig 1

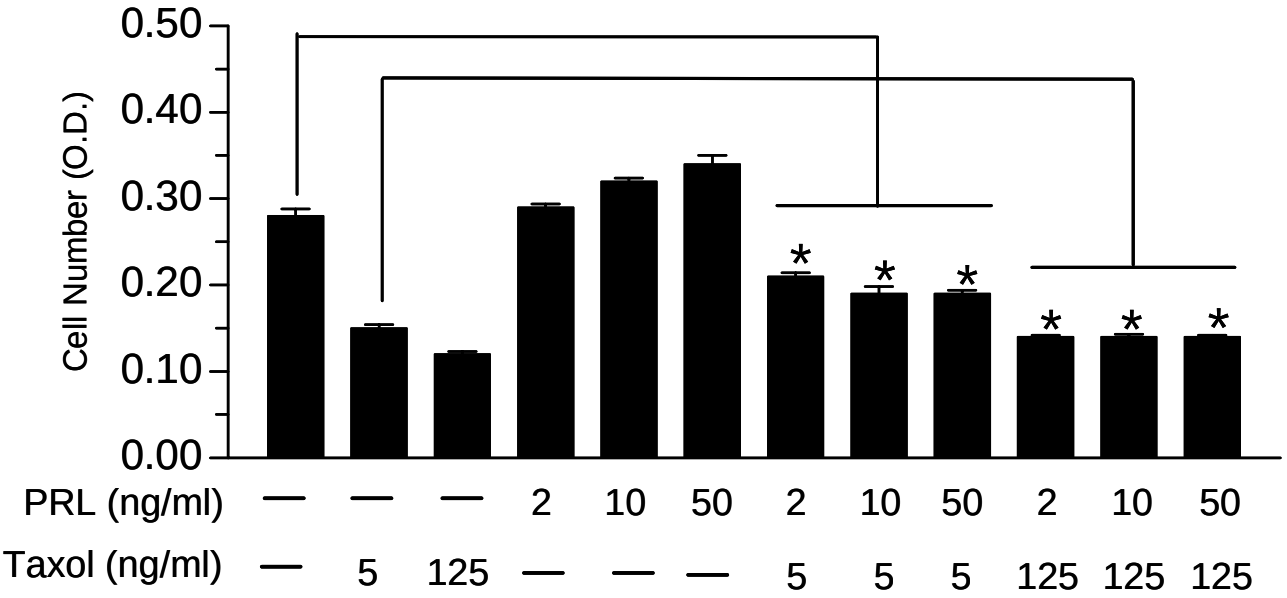
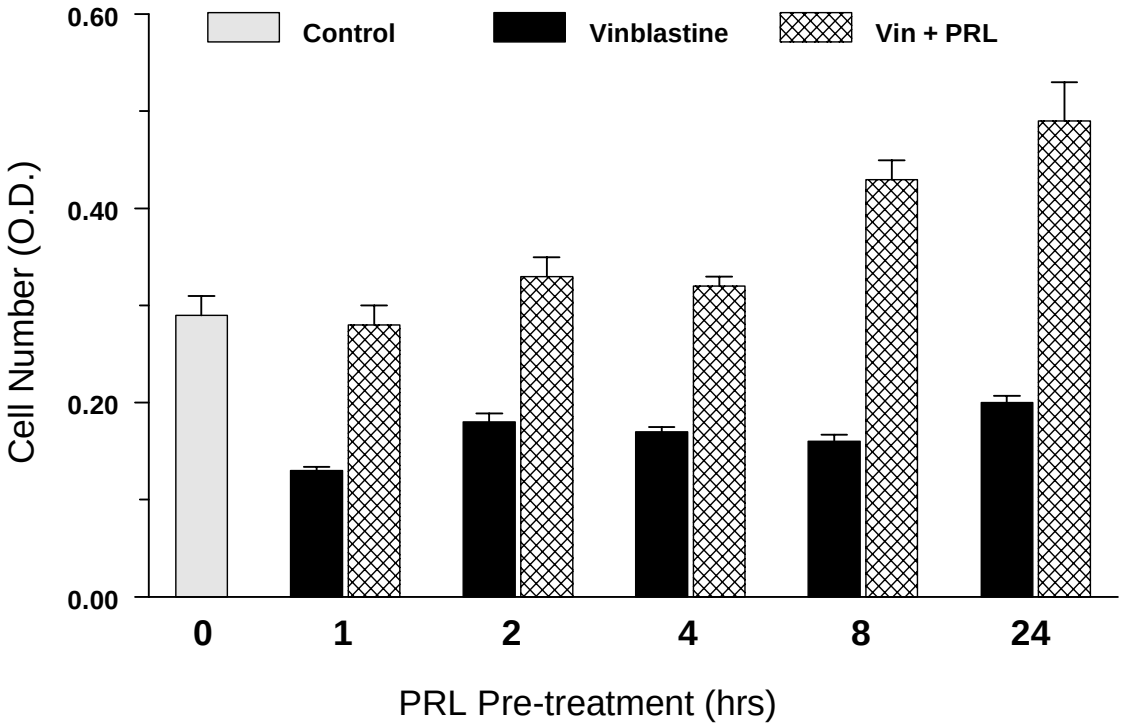

0.50
0.40
0.30
0.20
0.10
0.00
Cell Number (O.D.)
*
PRL (ng/ml) — — — 2 10 50 2 10 50 2 10 50
Taxol (ng/ml) — 5 125 — — — 5 5 5 125 125 125

Fig 2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Vinblastine
Vin + PRL
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0.20
0.00
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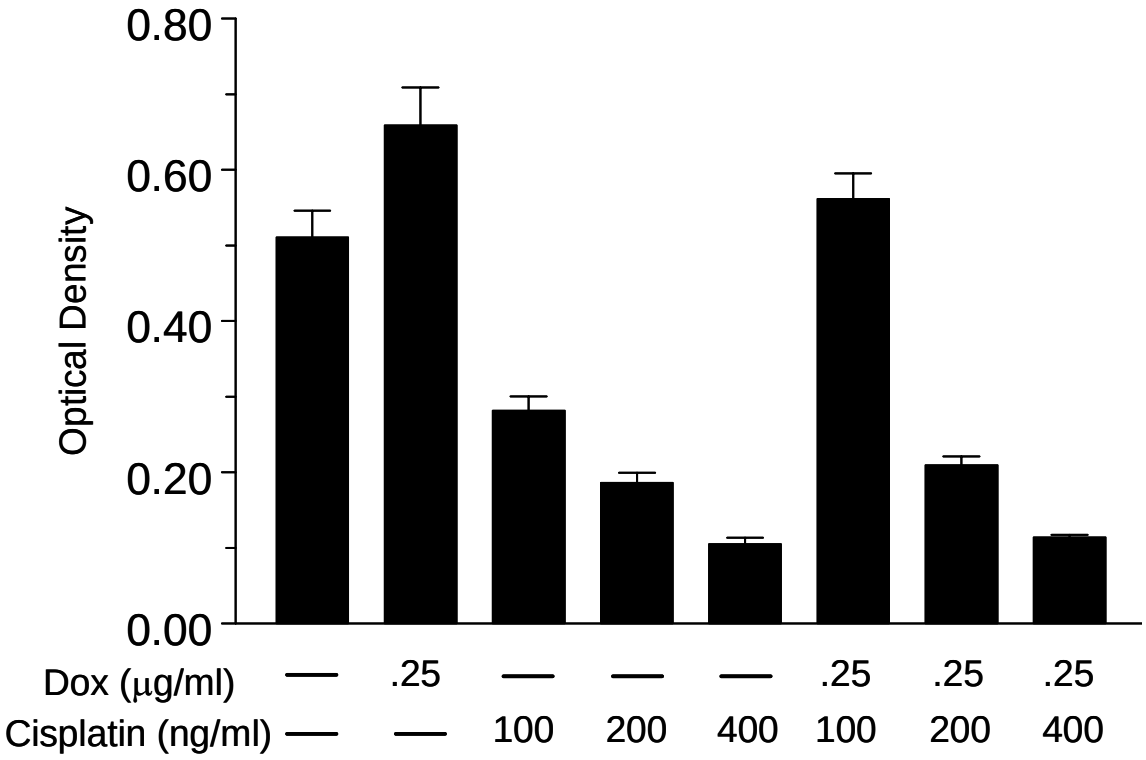
1
2
4
8
24
PRL Pre-treatment (hrs)

Fig 3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0.40
0.20
0.00
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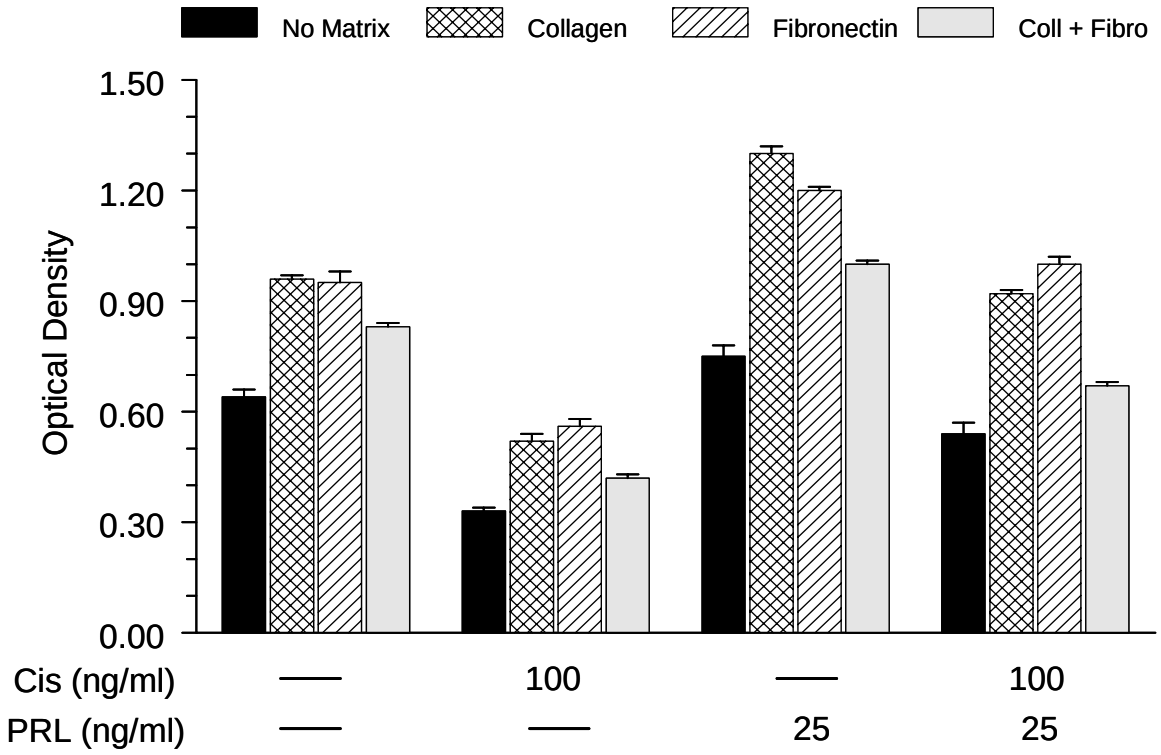
.25
.25
.25
Cisplatin (ng/ml)
100
200
400
100
200
400

Fig 4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Collagen
Fibronectin
Coll + Fibro
Optical Density
1.50
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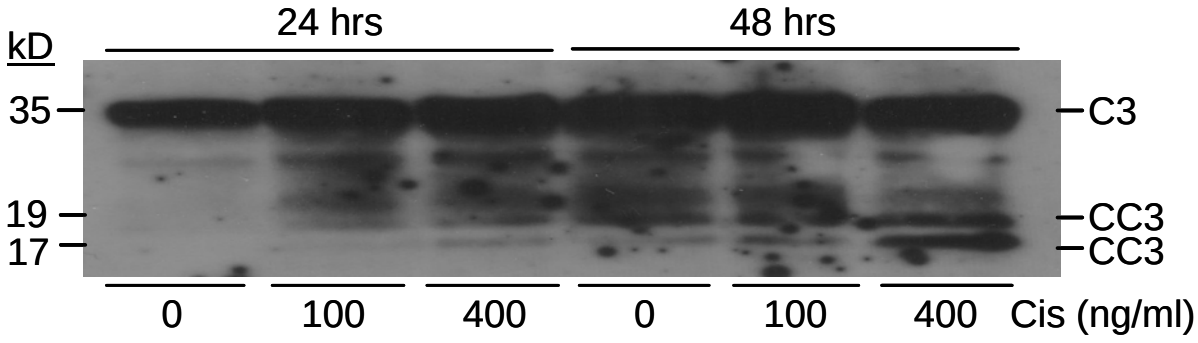
0.60
0.30
0.00
Cis (ng/ml)
PRL (ng/ml)
100
100
25
25

Fig 5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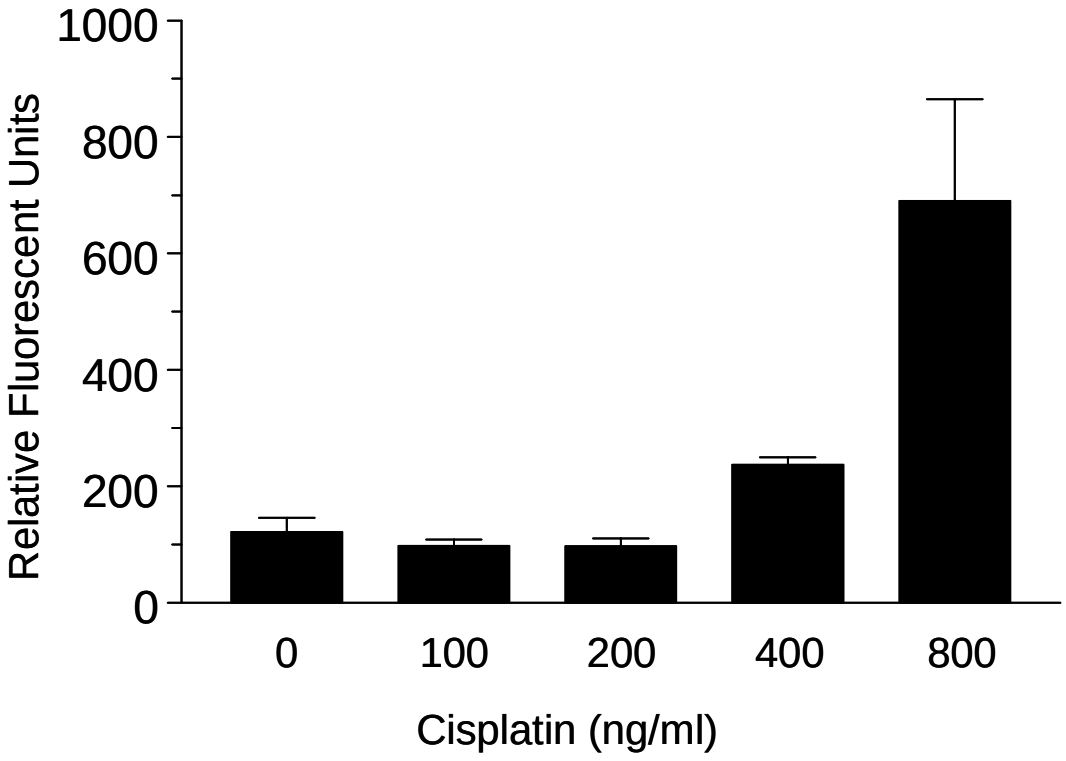
19
17
C3
CC3
CC3
0
100
400
0
100
400
Cis (ng/ml)

Fig 6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Relative Fluorescent Units
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100
200
400
800
Cisplatin (ng/ml)

Fig 7